TRAMP carcinogenesis is associated with a loss of COX expression and resistance to the tumor suppressive effects of omega-3 fatty acids.

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ABSTRACT

The "Western" dietary pattern found in affluent nations and characterized as high in lipid content, low in fiber, rich in red meat, and limited in fruits and vegetables is correlated with a greater risk of prostate cancer. In addition, it is hypothesized that diets rich in omega-3 fatty acids, such as found in many fish, may be preventive against prostate cancer. The most abundant and most thoroughly studied fatty acids found in fish oil are eicospentanoic acid (EPA) and docosahexanoic acid (DHA). Supplementation with these fatty acids has been shown to inhibit growth of prostate cancer cells in culture. It is hypothesized that these fatty acids exert chemopreventive effects through a suppression of PGE2 and enhancement of PGE3 synthesis, subsequently reducing proinflammatory signals and/or enhancing antiproliferative/proapoptotic signaling.

We have chosen to investigate the effects of a high fish oil diet on prostate cancer chemoprevention in vivo with the TRAMP model of murine prostate cancer. This transgenic mouse develops invasive adenocarcinoma of the prostate as a result of androgen-driven, prostate-specific expression of the SV-40 T/t antigen. Male mice (4 week old) were placed on semipurified diets containing 40% of calories from fish oil (HFO) or corn oil (HCO) for 26 weeks. Time to 2cm diameter palpable tumor was recorded. Upon sacrifice, tissues were collected and concentrations of tissue fatty acids and prostaglandins were obtained. Prostate tumorigenesis was not altered by a lipid source. However, prostatic lipid profiles mimicked dietary changes. In addition, prostate PGE2 was reduced by the HFO diet in wild-type mice, but no changes in the prostates of TRAMP mice. PGE3 was increased by HFO diet in both wild-type and transgenic mice.

The resistance of TRAMP mice to anticancer activity by omega-3 rich diets was further investigated. We observed that COX expression was significantly lost during progression of TRAMP carcinogenesis based upon western blots, mRNA expression, and immunohistochemistry. These studies suggest that the TRAMP model, with carcinogenesis induced by prostatic expression of SV40 T antigens disrupts fatty acid and lipid metabolism in a manor that produces resistance to dietary intervention.

In conclusion, the HFO diet did not prevent prostatic tumorigenesis in the TRAMP model but did affect levels of PGE2 and PGE3 and substrates for their metabolism.

INTRODUCTION

Epidemiologic and cell culture studies have suggested that the types of dietary lipids are associated with the risk of prostate cancer. In brief, diets rich in saturated fats and omega-6 fatty acids are hypothesized to increase risk while diets containing omega-3 fatty acids are proposed to reduce risk.

Arachidnonic acid, a predominate dietary omega-6 fatty acid, is metabolized through the cyclooxygenase (COX) enzyme pathways to prostaglandin E2 (PGE2). PGE2 levels have been found to be elevated in prostate cancer and is associated with aggressive tumor behavior and biomarkers of enhanced angiogenesis and invasiveness (2).

Diets rich in fish products contain much greater concentrations of omega-3 fatty acids. The most abundant and thoroughly studied of these fatty acids are eicospentanoic acid (EPA) and docosahexanoic acid (DHA). These fatty acids can inhibit the growth of culture prostate cancer cells. EPA and DHA have been shown to antagonize the effects of omega-6 fatty acids through COX-2, thereby decreasing levels of PGE2 (3). Moreover, increased omega-3 fatty acids can lead to increased production of the anti-inflammatory PGE3 (4).

We have chosen to investigate the effects of a a fish oil diet on prostate carcinogenesis in the TRAMP mouse model. TRAMP mice express an androgen driven SV-40 T/t transgene causing silencing of the p53 and Rb genes, which are common mutations seen in advanced prostate cancer. We also report the effects of consuming a fish oil diet on prostate fatty acid content, prostaglandin production, and the expression of COX enzymes during TRAMP carcinogesis.

METHODS

Animals- 4 week old male TRAMP mice were housed 5 per cage at 22±-0.5°C on a 12 hour day/night cycle. They were subjected to the standard AIN-76 diet or diets which provided 40% of calories from fish oil or corn oil for 26 weeks at which point they were sacrificed for analysis.

PG Analysis-Intracellular cell suspensions were washed withPBS and resuspended Aliquots of 1 N citric acid, 10% butylated hydroxytoluene (BHT), and PGE2-d4 (100 ng/ml) as an internal standard were added to the suspensions. Prostaglandins were extracted with hexane-ethyl acetate (1:1, v/v) three times. Samples were then reconstituted in methanol/10 mM ammonium acetate buffer (70:30, v/v), pH 8.5, before analysis by liquid chromatography/tandem mass spectrometry (LC/MS/MS).
The solution was applied to a Sep-Pak C18 cartridge (Waters Corp., Milford, MA) that had been preconditioned with methanol and water. Prostaglandins were eluted with methanol. The eluate was evaporated under a stream of nitrogen, and the residue was dissolved in methanol/10 mM ammonium acetate buffer (70:30, v/v), pH 8.5.
The extracted prostaglandins were quantitated by the LC/MS/MS method. LC/MS/MS was performed using a Quattro Ultima tandem mass spectrometer (Waters Corp., Milford, MA) equipped with an Agilent HP 1100 binary pump HPLC inlet (Agilent Technologies, Palo Alto, CA). The prostaglandins were separated using a 2 x 150 mm Luna 3 µ phenyl-hexyl analytical column (Phenomenex, Torrance, CA). The mobile phase consisted of 10 mM ammonium acetate, pH 8.5, and methanol. The column temperature was maintained at 50°C, and samples were kept at 4°C during the analysis. Individual analytes were detected using electrospray negative ionization and multiple reaction monitoring monitoring of the transitions *m/z* 351 271 for PGE2, *m/z* 349 269 for PGE3, and *m/z* 355 275 for PGE2-d4. Fragmentation of all compounds was performed using argon as the collision gas at a collision cell pressure of 2.10 x 10−3 Torr.

Fatty Acid Analysis-Dorsal prostate tissues were obtained from 30 week old TRAMP transgenic mice. Tissues were frozen in liquid nitrogen. Samples were homogenized, and subjected to two extractions using a 2:1 Chloroform: ice cold methanol/BHT (50ug/mL) solution 0.88% KCl was added to form the aqueous layer. The samples were then centrifuged for 10 minutes at 1000G, 4°C. Methanolysis reagent (1:4 TMG:methanol, v/v) was added to each sample and heated in a water bath. 0.88% KCl was added to each sample along with n-hexane. Two more extractions were performed, removing the upper aqueous phase. The n-hexane was evaporated from each sample under a strea nitrogen and in a RT water bath. The resulting sample was then reconstituted in n-he and analyzed by gas chromatography. Fatty acid methyl esters were analyzed by chromatography(HP5890 equipped with FID and 30-m Omegawax capillary column, Supelco Chromatography Products). Fatty acids were identified using authentic standards (Matreya), quantified by determining areas under identified peaks (ChemStation Software; Packard Instrument Company, Meriden, CT, USA).

RNA Extraction and Real-Time PCR (qRT-PCR)-Total RNA from mouse DP (WT, TRAMP at 10 and 16 wk of age and PD) was isolated by TRIzol reagent and further purified by RNeasy Mini kit as described by Qiagen. Both the quantity and quality of total RNA were analyzed by the Agilent Bioanalyzer 2100 system. Total RNA was reverse transcribed with an iScript cDNA synthesis kit. qRT-PCR was performed to determine the expression of COX-1 and COX-2 using a 1:10 dilution of cDNA with Taqman gene expression primers and ABI mastermix according to the manufacturer's instructions on an iCycler IQ qRT-PCR detection system (Bio-Rad). 18s RNA was used as the reference gene for all samples.

Immunoblotting-Total proteins were collected from frozen tissue by standard methods. Approximately 40 µg protein was electrophoresed on a 10% SDS-polyacrylamide gel followed by standard immunoblotting procedures. Four commercial sources of the primary antibodies against COX-2 were analyzed at the following dilutions: 1:500 (Santa Cruz), 1:750 (Cayman), 1:250 (BD) and 1:500 (Oxford). Two sources of the COX-1 antibodies were analyzed at dilutions of 1:500 for the Santa Cruz antibody and 1:1000 for antibody for the Cayman Chemical antibody. The secondary antibody was goat anti-rabbit IgG HRP for COX-1 (Cayman) and COX-2 (Cayman, BD and Oxford). The donkey anti-goat IgG HRP was used for COX-1 and COX-2 antibodies from Santa Cruz. Beta-actin was used as a loading control. Cell lysates from a wild type mouse vas-deferens was used as positive control for COX-1 and COX-2 immunoblotting.

OBJECTIVES

- To determine if diets rich in omega-3 fatty acids derived from fish oil modify prostate carcinogenesis in the TRAMP model
- To investigate the effects of diets rich in omega-3 fatty acids derived from fish oil on prostate prostaglandin production in control and TRAMP mice.
- To examine the expression of COX-I and COX-II during prostate carcinogenesis in TRAMP mice.

RESULTS

Dietary lipid composition has no effect on time to palpable tumor formation

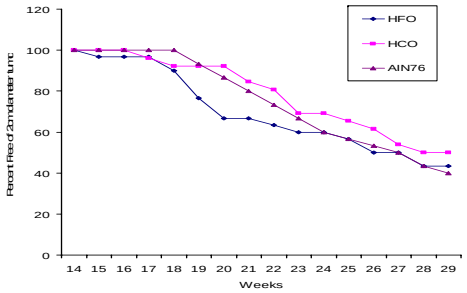

Figure 1 - The percentage of TRAMP mice free of a palpable prostate tumor (2 cm diameter) over time. Mice were examined weekly to asses the presence or absence of a palpable tumor.

Dietary lipid and genotype interactions on the prostate content of prostaglandin E3

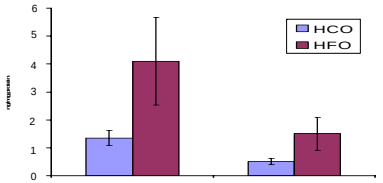

Figure 3 - The HFO diet increased levels of prostaglandin E3 in wild-type mice, which TRAMP mice show a significantly lower content, regardless of diet. Prostaglandin concentrations were determined using MS. N=6-7 per diet.

COX-1 and COX-2 gene expression is lost during TRAMP carcinogenesis – Western analysis

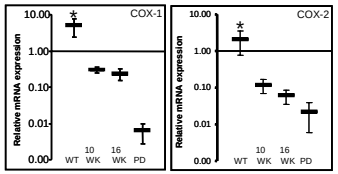

Figure 5-Western blot analysis of COX-1 and COX-2 protein levels in DP tissue from WT (10 wk), TRAMP DP (10 and 16 wk), and from histological confirmed poorly-differentiated tumor tissue (PD). Mouse vas deferens tissue was used as positive control for COX (+). Tissue lysates from two different mice were analyzed for each tissue type and images are representative of two independent experiments.

Dietary lipid and genotype interactions on the prostate content of prostaglandin E2

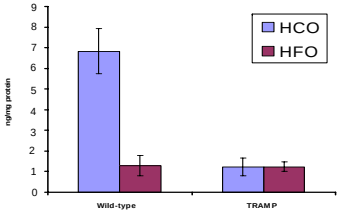

Figure 2 - Prostaglandin E2 production in the prostates of wild-type mice is reduced by diets rich in fish oil, but has no effect in TRAMP mice. Prostaglandin levels were determined using MS. N=6-7 per diet

Diet alters prostatic fatty acid pattern

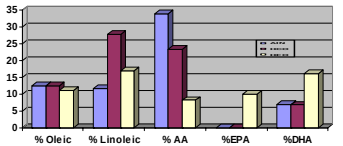

Figure 4 - Prostatic fatty acid pattern mimics dietary intake. Relative fatty acid patterns were determined using the Folch method of lipid extraction and gas chromatography. N=3 per diet. HFO diet decreases the % of arachidonic acid and increases the % DHA and EPA.

COX-1 and COX-2 gene expression is lost during TRAMP carcinogenesis – RT-PCR analysis

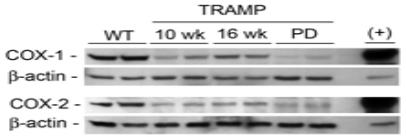

Figure 6-Quantitative RT-PCR of COX-1 and COX-2 mRNA levels in WT (10 wk) and TRAMP DP tissues (10 and 16 wk), and PD tumor. Each bar represents the mean COX-1 or COX-2 mRNA expression (error bars are 95% confidence intervals) of five animals relative to a common calibrator sample generated by pooling the dorsal, ventral, and lateral prostates of a single wild-type mouse. 18s RNA levels were used to normalize the expression of individual samples. Data points with an asterisk are significantly different from other data points in the same panel ($p < 0.05$).

CONCLUSIONS

- A diet rich in fish oil diet does not inhibit prostate carcinogenesis in the TRAMP model
- Prostate fatty acids are changed in response to patterns of fatty acids found in the diet
- Prostaglandin synthesis is affected by dietary fatty acid content in wild-type mice but not in the TRAMP model
- TRAMP carcinogenesis is assocaited with a loss of COX expression.
- The loss of COX expression in TRAMP results in a resistance to the chemopreventive effects of a an omega-3 fatty acid rich diet. This works suggests that disruptions in p53 and RB pathways alters the cellular response to fatty acids and PGs.

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